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DEPARTMENT OF TRADE AND INDUSTRY

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This is to certify that the documents attached hereto are true and correct copies of the Forms P2, P6, provisional specification of South African Patent Application No. 99/4176 in the name of UNIVERSITY OF PRETORIA

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Entitled : TREATMENT AND CONTROL OF TUBERCULOSIS

PRIORITY DOCUMENT

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in the Republic of South Africa, this day of

Registrateur van Patente
Registrar of Patents

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FORM P.2

REPUBLIC OF SOUTH AFRICA | REGISTER OF PATENTS | PATENTS ACT, 1

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FULL NAME(S) OF APPLICANT(S)/PATENTEE(S)

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PRIORITY CLAIMED	COUNTRY	NUMBER	DATE
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TITLE OF INVENTION

54 TREATMENT AND CONTROL OF TUBERCULOSIS

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FRESH APPLICATION BASED ON	DATE OF ANY CHANGE

SPOOR AND FISHER

FORM P.6

REPUBLIC OF SOUTH AFRICA
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PROVISIONAL SPECIFICATION

(Section 30(1) – Regulation 27)

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FULL NAME(S) OF APPLICANT(S)

71	UNIVERSITY OF PRETORIA

FULL NAME(S) OF INVENTOR(S)

72	JACOBUS JOHANNES MARION MEYER; NAMRITA LALL

TITLE OF INVENTION

54	TREATMENT AND CONTROL OF TUBERCULOSIS

BACKGROUND OF THE INVENTION

THIS invention relates to the treatment and control of tuberculosis caused by *Mycobacterium tuberculosis* and in particular to the use of naphthoquinone derivatives for use in such treatment and control.

Tuberculosis (TB) remains a serious health problem in many regions of the world, especially in developing nations. It is a contagious disease and is becoming epidemic in some parts of the world. It is estimated that 30-60% of adults in developing countries are infected with *Mycobacterium tuberculosis*. Approximately 8-10 million individuals develop clinical TB and 3 million die of TB each year (WHO/IUATLD, 1989).

In South Africa, over 3 in every thousand people die of TB, the highest rate in the world, while one out of every 200 people suffers from active tuberculosis. Tuberculosis is the most commonly notified disease in South Africa and the fifth largest cause of death among the black population (South African Tuberculosis Association, 1998).

In the United States, the number of TB cases steadily decreased until 1986 when an increase was noted. Since then TB cases have continued to rise. Ten million individuals are infected in the U.S.A., with approximately

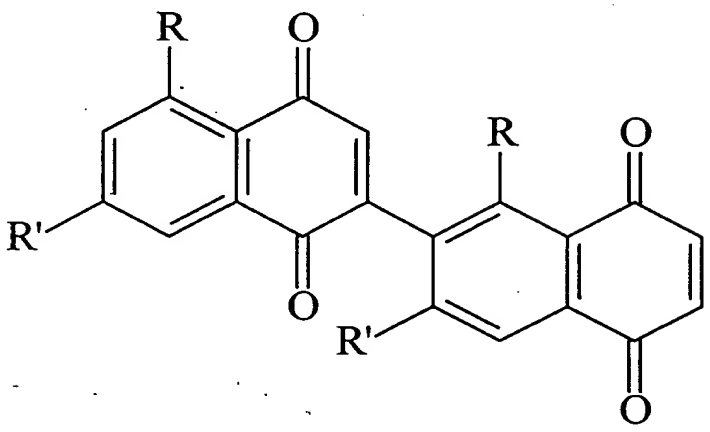
26000 new cases of active disease each year (National Jewish Medical and Research Centre, 1994).

Individuals infected with Human Immunodeficiency Virus (HIV) are very susceptible to tuberculosis and often develop this disease before other manifestations of AIDS become apparent (Grange and Davey, 1990). Control of the TB epidemic linked with HIV infection will depend largely on the adequate treatment of TB, and possibly of effective chemoprophylaxis, not just for HIV-infected persons but for communities as well (WHO/IUATLD, 1989).

TB therapy has been revolutionized and the present treatment regimes for TB are based on multidrug therapy with usually 3 or 4 antituberculosis drugs. However, the problem of multidrug resistant tubercle bacilli is emerging for various drugs such as isoniazid, ethambutol, rifampicin and streptomycin, for example (Girling, 1989; Grange and Davey, 1990). Drug-resistant TB is very difficult to treat requiring greater numbers and varieties of medications for a longer period of treatment. The need for new antituberculosis agents is urgent due to the increasing resistance of mycobacteria to these classic antituberculosis drugs. A recent WHO report states that, globally, 2% of all cases of tuberculosis are multidrug resistant - by definition, resistance to rifampicin plus isoniazid (plus/minus other resistances). Such cases can be treated in the USA and other high resource regions but at a great cost (> US$ 250,000 per case!) and using very long courses of rather toxic drugs, thereby raising serious problems of compliance (WHO, 1997). South Africa is witnessing an explosion in the number of cases of drug-resistant tuberculosis. In some parts of South Africa, 1 in 10 cases of TB is resistant to treatment (New Scientist, March 1997). It is essential to have new antituberculosis agents, preferably those that can readily and simply be produced from some local source.

SUMMARY OF THE INVENTION

According to the present invention there is provided a naphthoquinone derivative of Formula 1:



wherein,

R represents an OH group, methyl ether, ethyl ether or a similar ether; and

R' represents a methyl, ethyl or similar aliphatic hydrocarbon derivative,

or a pharmaceutically acceptable salt thereof for use in a method of treating and/or controlling tuberculosis in a patient caused by *Mycobacterium tuberculosis*.

The invention extends to the use of a naphthoquinone derivative of Formula 1 in the manufacture of a medicament for use in a method of treating and/or controlling tuberculosis in a patient caused by *Mycobacterium tuberculosis*.

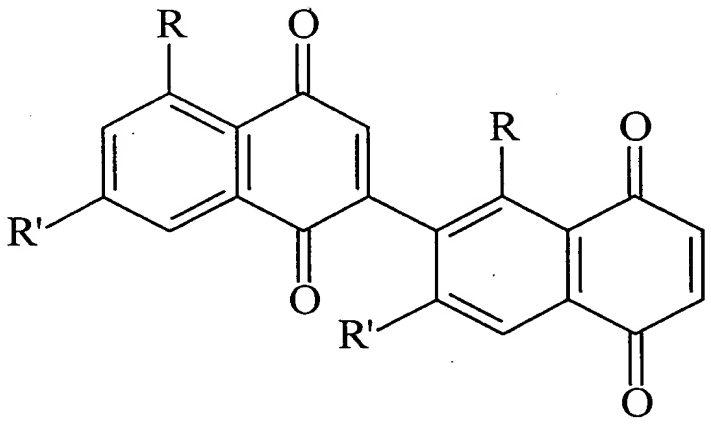
R in the compound of Formula 1 is preferably an OH group.

R' in the compound of Formula 1 is preferably a CH_3 group.

In particular the naphthoquinone derivative of Formula 1 is 5,5' dihydroxy 7,7' binaphthoquinone (diospyrin).

DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention is directed at the use of naphthoquinone derivatives in the treatment and/or control of tuberculosis caused by *Mycobacterium tuberculosis*. In particular, naphthoquinone derivatives of the general Formula 1 have been found to be effective against *Mycobacterium tuberbulosis*.



FORMULA 1

In particular diospyrin, a naphthoquinone derivative of Formula 1 in which R is OH and R' is a methyl group, has been found to inhibit several antibiotic resistant as well as antibiotic susceptible strains of *Mycobacterium tuberculosis*. Although diospyrin is particularly preferred, naphthoquinone derivatives of Formula 1 in which R is a methyl ether, ethyl ether or similar ether and R' is an ethyl or similar aliphatic hydrocarbon derivative, are also provided.

The inventors of the present application undertook an extensive research program in order to identify anti-tuberculosis agents that can readily and simply be produced from local resources.

Twenty South African medicinal plants used to treat pulmonary diseases were screened by the inventors for activity against drug-resistant and sensitive strains of *M. tuberculosis*. A preliminary screening of acetone and water plant extracts, against a drug-sensitive strain of *M. tuberculosis*; H37Rv, was carried out by the agar plate method. Fourteen of the 20 acetone extracts showed inhibitory activity at a concentration of 0.5 mg/ml against this strain. Acetone as well as water extracts of *Cryptocarya latifolia*, *Euclea natalensis*, *Helichrysum melanacme*, *Nidorella anomala* and *Thymus vulgaris* inhibited the growth of *M. tuberculosis*. Given the activity of 14 acetone extracts at 0.5 mg/ml against the drug-sensitive strain by the agar plate method a further study was carried out employing the rapid radiometric method to confirm the inhibitory activity. These active acetone extracts were screened against the H37Rv strain as well as a strain resistant to the drugs, isoniazid and rifampin. The minimal inhibitory concentration of *Croton pseudopulchellus*, *Ekebergia capensis*, *Euclea natalensis*, *Nidorella anomala* and *Polygala myrtifolia* was 0.1 mg/ml against the H37Rv strain by the radiometric method. Extracts of *Chenopodium ambrosioides*, *Ekebergia capensis*, *Euclea natalensis*, *Helichrysum melanacme*, *Nidorella anomala* and *Polygala myrtifolia* were active against the resistant strain at 0.1 mg/ml. Eight plants showed activity against both the strains at a concentration of 1.0 mg/ml.

The following procedure was developed by the applicant for the isolation of diospyrin from *E. natalensis* and other species in this genus, as well as any other plants that may synthesise diospyrin or other quinone derivatives.

1. **Identification of plant species**

Roots and the aerial plant parts of *E. natalensis* were collected near Durban and identified at the HGWJ Schweickerdt Herbarium of the University of Pretoria and also at the herbarium of the National Botanical Institute, Pretoria.

2. **Extraction**

Dried roots of *E. natalensis* were ground to a powdery form with a dry mill and extracted over 48 hours with acetone. The extract was filtered and concentrated to dryness at reduced pressure on a rotary evaporator.

3. **Thin layer chromatography**

A direct antibacterial bioassay (Dilika & Meyer 1996) on TLC-plates was employed to speedup the activity guided isolation of the antituberculosis compound. *M. tuberculosis* cannot be tested in this way because of its very slow growth rate. The direct antibacterial bioassays of the acetone extract were done on TLC plates (Merck) developed with chloroform-hexane (1:1). After development, the TLC plates were dried and sprayed with a 24 hr old *Staphylococcus aureus* culture in nutrient broth. After 24 hr incubation, the plates were sprayed with an aqueous solution of 2mg/ml p-iodonitrotetrazolium violet to visualise the bacterial cells. The plates were then reincubated at 37^0C for 2-3 hours.

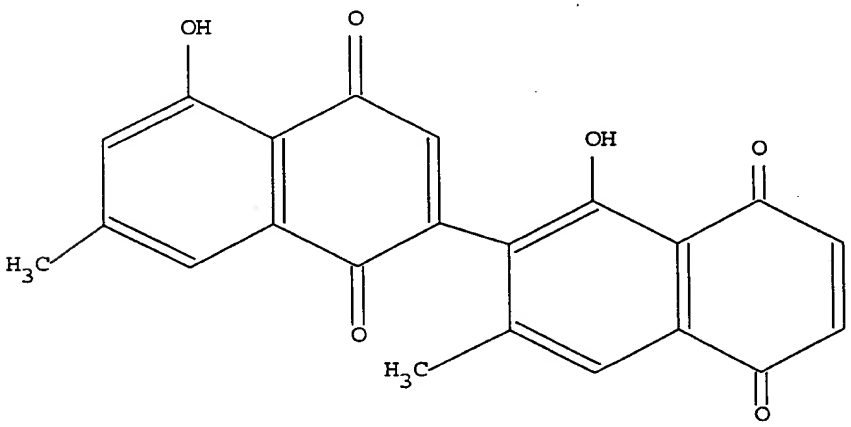
Two zones of bacterial growth inhibition could be seen on TLC plates sprayed with *S. aureus*. Activity was more pronounced in the R_f 0.30 zone (chloroform-hexane (1:1)) than in the R_f 0.54 zone.

4. Column chromatography

The crude extract of the plant was dried, its mass determined and resuspended in chloroform. Column chromatography was performed on silica gel 60 using chloroform as eluent. The antibacterial fractions collected were then subjected to a Sephadex LH-20 column chromatography using ethanol as eluent. The fractions collected were again tested for antibacterial activity on TLC to detect the fraction containing the active compound of R_f 0.30.

5. High performance liquid chromatography

The compound was further purified by HPLC utilising an analytical Phenomenex reverse phase 250x4.60 mm column, at a flow rate of 1.0 ml/min, oven temp. 40^0C and a wavelength of 206nm. An ethanol-water (50:50) solution was employed as mobile phase. The pure compound was once again subjected to a Sephadex LH-20 column chromatography and proved to be pure. The chemical structure was confirmed by ^{1}H and ^{13}C nmr and ms to be:



Diospyrin (5,5' dihydroxy 7,7' binaphthoquinone); $C_{22}H_{14}O_6$. Molecular weight: 374.

The effect of diospyrin on the growth of the sensitive strain (H37Rv) and resistant strains of *Mycobacterium tuberculosis* as determined by the radiometric method are set out in Table 1.

TABLE 1

Mycobacterium tuberculosis strains	MIC (mg/ml)	ΔGI[a] values of plant extracts (mg/ml)	ΔGI values of the control vial (mg/ml)
H37 sensitive strain	0.1	-1 ± 1.41	20 ± 4.24
2 drug resistant strain (res. to Isoniazid and rifampicin).	0.1	3.5 ± 0.70	25 ± 7.07
3 drug resistant strain (res. to streptomycin, isoniazid and ethambutol),	0.1	4 ± 2.12	29 ± 1.41
4 drug resistant strain (res. to streptomycin, isoniazid, rifampicin and ethambutol).	0.1	5 ± 2.82	25 ± 2.82
5 drug resistant strain.(res to isoniazid, streptomycin, rifampicin, thiacetazone and cyclocerine).	0.1	10 ± 1.41	22.5 ± 3.53
6 drug resistant strain (res. to isoniazid, rifampicin, ethionamide, terizidone, thiacetazone and ofloxacin).	0.1	9 ± 2.82	30 ± 1.0
7 drug resistant strain.(res to isoniazid, steptomycin, ethambutol, kanamycin, rifampicin, and ethionamide)	0.1	13.5 ±3.2	28 ± 3.1

[a] ΔGI values are means ± s.d.

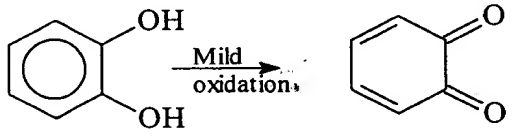
The results show that diospyrin controls the *Mycobacterium tuberculosis* bacterium effectively. Oral administration of diospyrin in an appropriate pharmaceutical composition with suitable diluents and carriers will typically

be used to treat or control tuberculosis. This will be by way of tablet, liquid or similar oral dosage form, as dispyrin is readily absorbed intestinally.

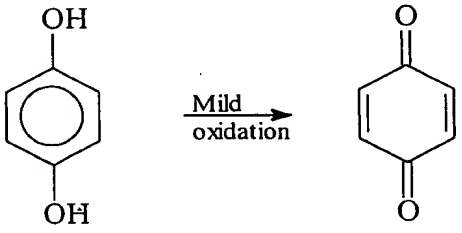
However, it is believed that diospyrin administered intravenously or intramuscularly will also be absorbed effectively through blood vessels and the blood stream of a patient. Transdermal administration, via a plaster or similar transdermal administration vehicle, is also a possibility.

The Applicant believes that it may be possible to increase the concentration of diospyrin and other quinones in *E. natalensis* or similar species by phytoalexic stimulation or by the biotechnological manipulation of tissue cultures and/or intact plants.

Quinones are generally synthesised from catechol (1,2-quinones) or hydroquinone (1,4-quinones) by mild oxidation.

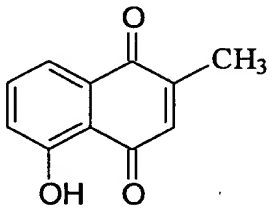


Catechol



Hydroquinone

As far as the applicant has been able to establish, diospyrin has never been synthesised in a laboratory. However, similar binapthoquinones can be synthesised by the reaction of plumbagin (94mg in methanol, 10ml) and its hydroquinone (190mg in methanol, 14ml), buffered in phosphate to pH 6.8 at 30°C. (Sankaram et al. 1975; Kumari et al. 1982).



Plumbagin

It is believed that diospyrin and related naphthoquinone derivatives are viable alternatives to conventional drugs in the treatment and control of tuberculosis in humans.

DATED THIS 24TH DAY OF JUNE 1999

SPOOR AND FISHER
APPLICANTS PATENT ATTORNEYS